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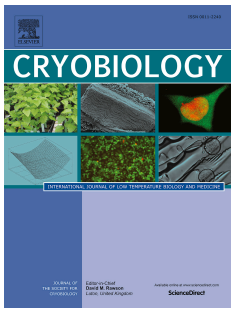

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Md Mahbubul Hassan, Xiaoxu Li, Jian G. Qin

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Improvement of post-thaw sperm survivals using liquid nitrogen vapour in a spermcasting oyster *Ostrea angasi*

Md Mahbubul Hassan[a,c], Xiaoxu Li[b*] and Jian G. Qin[a]

[a]School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia

[b]Aquatic Sciences, South Australian Research and Development Institute, 2 Hamra Avenue, West Beach, SA 5024, Australia

[c]Department of Fisheries Biology and Genetics, Hajee Mohammad Danesh Science and Technology University, Dinajpur 5200, Bangladesh

*Corresponding author

Email: xiaoxu.li@sa.gov.au

Abstract

Low survival of cryopreserved sperm impedes the application of cryopreservation technique in spermcasting oyster species. This study developed a simple method of liquid nitrogen vapor freezing to improve post-thaw sperm survival in the spermcasting oyster *Ostrea angasi*. The results indicate that the permeable cryoprotectants, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG) were non-toxic to sperm up to 20% concentration and 90 min exposure whereas methanol at 10% or higher was toxic to sperm for any exposure over 30 min. Among the treatments with permeable cryoprotectants, 15% EG produced the highest post-thaw sperm motility. Sperm motility was further improved by the addition of non-permeable cryoprotectants (trehalose and glucose), with 15% EG + 0.2 M trehalose resulting in the highest post-thaw sperm motility among all the combinations evaluated. The durations of 20, 30 and 60 min equilibrations produced a higher post-thaw sperm motility and plasma membrane integrity (PMI) than 10 min. Higher post-thaw motility and PMI were achieved by freezing sperm at the 8 cm height from the liquid nitrogen surface than at the 2, 4, 6, 10 or 12 cm height. Holding sperm for 10 min in liquid nitrogen vapor produced higher post-thaw motility and PMI than for 2, 5 or 20 min. The cryopreservation protocol developed in this study improved both post-thaw motility and PMI of *O. angasi* sperm at least 15% higher than those cryopreserved using programmable freezing method. Liquid nitrogen vapour freezing might have greater applicability in improving post-thaw sperm quality of spermcasting oyster species.

Keywords: Cryopreservation, freezing, sperm survival, mollusc

Introduction

Cryopreservation is a promising method for storage of important genotypes in the application of aquaculture biotechnology. In the past 50 years, sperm cryopreservation research in marine invertebrates has made substantial progress in marine aquaculture [18, 32]. Among marine invertebrates, the edible oysters are most widely studied [36], but 95% of the work has focused on broadcasting species [18]. The post-thaw sperm motility achieved in spermcasting species is low [21, 39] compared to broadcasting species [13, 41] probably because of the difference in their spermatological characteristics. The spermcasting species release clusters of spermatozeugma [19] whereas broadcasting species release individual sperm. In the first sperm cryopreservation study of the Australian flat oyster, 44% post-thaw motility and 49% plasma membrane integrity (PMI) were achieved using a programmable freezing method [17]. Therefore, sperm quality needs to be further improved to increase the efficiency of cryopreservation in future breeding and genetic improvement programs in spermcasting species.

Different freezing methods have been successfully used for cryopreservation such as programmable computer controlled freezing and liquid nitrogen vapor freezing. Even though the programmable freezing is a widely-used method for sperm cryopreservation, the liquid nitrogen vapor method has been successfully applied in many marine invertebrates including the Pacific oyster [42], green-lip abalone [43], greenshell mussel [37], and pearl oyster [29]. The liquid nitrogen vapour freezing method has been found to be a better option to establish a gamete cryobanking service to the Australian aquaculture industry due to low initial investment and easy farm accessibility [24]. Freezing with liquid nitrogen vapor does not require expensive equipment and highly skilled personnel. As a large quantity of sperm can be cryopreserved within a short time in a single batch, this method is considered simple, less

63 expensive and efficient. However, the application of liquid nitrogen vapor method to
64 cryopreserve the Australian flat oyster sperm has not yet been evaluated.

65 The quality of sperm can be compromised by the cryopreservation process. However,
66 optimization of the steps in cryopreservation can improve the ability of sperm to withstand
67 damages from cryopreservation. Although cryoprotectants could minimize freezing and
68 thawing injury, they may also be toxic to sperm at a high concentration. Therefore, the
69 evaluation of the sperm tolerance to cryoprotectants should be the first step for cryoprotectant
70 selections. The cryoprotectants consist of two types of chemicals: the permeable and non-
71 permeable cryoprotectants. The former enters into the cell and bring an equilibrium between
72 extracellular and intracellular solutes whereas the latter stabilizes the cell membrane and
73 increase cell membrane cohesiveness [11]. Therefore, suspending sperm with both types of
74 cryoprotectants may minimize the chance of intracellular ice formation and cell membrane
75 shrinkage. Freezing is the most important step in a cryopreservation procedure because most
76 sperm injuries occur within the temperature range of 0 °C - 40 °C due to the formation of
77 intracellular ice. In liquid nitrogen vapor freezing method, the distance of the sample from the
78 liquid nitrogen surface, holding duration of samples in liquid nitrogen vapor and straw size
79 can all affect the application of the cryopreservation protocol and need to be optimised. This
80 study aimed to develop a non-programmable freezing technique to improve the post-thaw
81 sperm quality of the Australian flat oyster, including the optimization of cryoprotectant
82 concentration, equilibration duration, sperm distance to liquid nitrogen surface, holding
83 duration, and sperm volume.
84

Materials and methods

The oysters

Pristine Oyster Farm in Coffin Bay, South Australia provided the two years old mature flat oysters (76.8 ± 4.4 mm in shell length and 71.2 ± 11.8 g in total weight). The oysters were shipped to South Australian Research and Development Institute in a chilled Styrofoam box during September and November, 2014. The oysters were cleaned off debris and epifauna, and placed in tanks supplied with flow-through seawater and aeration. Oysters were maintained in tanks to facilitate sperm collection. Mixed microalgae of *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans* were supplied to the tank and water temperature was maintained at 20 ± 0.5 °C.

Freezing apparatus

A Styrofoam box (39.0 × 24.5 × 35.5 cm) and foam racks of different heights (2, 4, 6, 8, 10 and 12 cm) were used in this study. Approximately 4-cm liquid nitrogen was placed in the Styrofoam box and the lid was loosely closed to expel air for 3 min before placing the straws on a rack at the required height from the liquid nitrogen surface.

Cryopreservation protocol

The following general cryopreservation protocol was used in all the experiments (except otherwise indicated). Sperm were equilibrated with a cryoprotectant for 20 min, packaged in 0.25 ml French straws, placed on a foam rack of 8 cm above the liquid nitrogen surface for 20

min, plunged into liquid nitrogen, and then stored in the liquid nitrogen dewar. The cryopreserved sperm were thawed in 40 °C water for 6 sec prior to motility evaluation.

Sperm collection and quality assessment

The details of sperm collection and quality assessment for this flat oyster species have been described by Hassan et al [17]. Briefly, male oysters were identified by microscopic observation of stripped gametes. Gametes were collected by stripping with a 3.5 ml Pasteur pipette and placed on ice until being used in the study. The stripped gonad samples contain spermatozeugmata (average diameter 117 µm) which starts to dissociate into individual sperm after collection [19]. Collected sperm were filtered through a 45-µm screen, and the sperm concentration was adjusted to 1×10^9 cells/ml after being counted with the spectrophotometric method [20]. The samples with at least 50% sperm motility were used in all the experiments. Sperm were pooled from 3 to 5 males in each replicate. Sperm motility was blindly assessed by two observers using a light microscope and also by video recording using an Olympus BX60 microscope with a 10x objective lens. An aliquot of 2 µl sperm was placed on a glass slide and then diluted with 20 µl filtered seawater for motility assessment by direct observation and recording. Video recording lasted for 30 sec. Sperm showing forward movement in consecutive video frames were considered motile. At least 50 randomly selected sperm were analysed from each video clip. The motility percentage from two observers was averaged as mean sperm motility of the subsamples and was validated (only if higher than 10% variation) by video-recorded motility. To analyse PMI, dual staining by SYBR 14 and propidium iodide was used to distinguish live and dead sperm under an Olympus BX60 fluorescence microscope.

Experiment 1: Cryoprotectant toxicity

Four permeable cryoprotectants dimethyl sulfoxide (DMSO), methanol, ethylene glycol (EG) and propylene glycol (PG) were used to evaluate cryoprotectant toxicity. Sperm were equilibrated with each cryoprotectant at 5, 10, 15 and 20% final concentrations for 30, 60 and 90 min, respectively. Sperm motility was used as a toxicity assessment indicator.

Experiment 2: Cryoprotectant selection

In the first trial, the effect of a permeable cryoprotectant on post-thaw sperm motility was evaluated. Sperm were equilibrated with 5, 10, 15 and 20% of DMSO, methanol, EG and PG at 4°C for 30 min, and then cryopreserved. In the second trial, glucose and trehalose were added to DMSO and EG to evaluate the combination effects of permeable and non-permeable cryoprotectants on post-thaw sperm motility. A total of 18 cryoprotectant combinations were evaluated, including 10% DMSO, 10% EG or 15% EG in combination with 0.2, 0.4 or 0.6 M glucose or trehalose. In the third trial, sperm were equilibrated with 15% EG + 0.2 M trehalose for 10, 20, 30 or 60 min before being frozen to evaluate the effect of equilibration duration on post-thaw sperm motility and PMI.

Experiment 3: The effects of rack height

Based on the results of experiment 2, sperm were equilibrated with 15% EG + 0.2 M trehalose for 20 min in the following experiments. Equilibrated sperm was placed on a foam rack at 2, 4, 6, 8, 10 or 12 cm above the surface of liquid nitrogen to evaluate the effect of rack heights on post-thaw sperm motility and PMI. Temperatures at different rack heights were recorded with a digital thermometer (Thermo Scan, Eutech Instruments, Singapore) attached to a temperature probe. The average cooling rates for rack heights of 2, 4, 6, 8, 10 and 12 cm were - 74.3, - 48.7, - 34.6, - 25.8, - 21.3 and - 16.9 °C/min, respectively.

Experiment 4: The effects of holding duration

Sperm were held on the rack 8 cm above the liquid nitrogen surface for 2, 5, 10 and 20 min to evaluate the effect of holding duration on post-thaw sperm motility and PMI.

Experiment 5: The effects of sperm volume

The effects of sperm volume on post-thaw motility and PMI were evaluated by freezing sperm in 0.25 ml and 0.5 ml straws. The 0.25 ml and 0.5 ml straws were thawed in a 40 $^{\circ}$C water bath for 6 sec and 8 sec, respectively.

Experiment 6: Comparison of freezing methods

The programmable freezing and liquid nitrogen vapor freezing methods were compared based on post-thaw sperm motility and PMI achieved in each method. The protocol of programmable freezing method was obtained from Hassan et al. [17]. The programmable freezing protocol includes: sperm were equilibrated in 10% DMSO + 0.45 M trehalose for 30 min, packaged in 0.5 ml straws, frozen at -3 ^{o}C/min, and thawed at 40 ^{o}C for 8 sec.

Statistical analysis

To standardize sperm motility in different treatments for statistical analysis, fresh motility was set to 100 percent and post-thaw sperm motility was calculated relative to fresh motility. However, percentages of observed sperm motilities are presented in figures and results. All the percentage data were arcsine transformed prior to statistical analysis. Depending on the number of factors involved, treatment effects on sperm motility and PMI in experiments 1 to 4 were analysed with one-factor or two factors analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) when differences were

significant. A repeated-measure design was applied when data were collected at different time intervals. In experiments 5 and 6, treatment effects on post-thaw sperm motility and PMI were analysed with independent t-test. Data were presented as mean ± SE. The significant level was set at $P < 0.05$. Data were analysed with SPSS version 20.0 (IBM Corporation, Armonk, NY, USA).

Results

Experiment 1: Cryoprotectant toxicity

Motility of sperm exposed to DMSO, EG and PG was similar at all the concentrations and exposure durations evaluated (Fig 1). Sperm motility (%) decreased after 30 min exposure to 10% or higher methanol, and the toxicity of methanol increased with increasing concentration and exposure time.

Experiment 2: Cryoprotectant selection

The post-thaw motility of sperm cryopreserved with different cryoprotectant concentrations were significantly different ($P < 0.05$). The highest post-thaw motility was achieved by cryopreserving sperm with 15% EG, followed by 10% EG and 10% DMSO (Fig 2A). Sperm cryopreserved with other cryoprotectant concentrations had significantly low post-thaw motility ($P < 0.05$).

The inclusion of glucose and trehalose in DMSO or EG solutions significantly increased the post-thaw sperm motility ($P < 0.05$). The highest motility (%) was achieved by cryopreserving sperm with 15% EG + 0.2 M trehalose, followed by 15% EG + 0.2 M glucose and 15% EG + 0.4 M trehalose (Fig 2B).

The highest post-thaw motility and PMI were achieved in this experiment by equilibrating sperm for 20 min (Fig 3). Sperm cryopreserved at 10 min equilibration

211 produced significantly lower post-thaw motility and PMI than 20, 30 and 60 min ($P < 0.05$).
212 The post-thaw motility and PMI of sperm cryopreserved at 20, 30 and 60 min equilibration
213 were similar.
214
215 *Experiment 3: Effect of rack heights*
216 The highest post-thaw sperm motility (%) and PMI were achieved in this experiment
217 by placing sperm on the 8 cm rack (Fig 4). All the other rack heights used in this experiment
218 produced significantly low post-thaw motility and PMI than the 8 cm rack height ($P < 0.05$).
219
220 *Experiment 4: Effect of holding durations*
221 The highest post-thaw motility and PMI were achieved in this experiment by holding
222 sperm in liquid nitrogen vapor for 10 min or 20 min, and both durations were significantly
223 different from 2 min and 5 min ($P < 0.05$; Fig 5).
224
225 *Experiment 5: Effect of straw volume*
226 Sperm packed in both 0.25 ml and 0.5 ml straws resulted in a similar post-thaw
227 motility and PMI ($P > 0.05$; Fig 6).
228
229 *Experiment 6: Comparison of freezing methods*
230 The post-thaw sperm motility and PMI were affected by the freezing methods
231 evaluated. The post-thaw sperm motility and PMI achieved by the liquid nitrogen vapor
232 freezing method were significantly higher than the programmable freezing method ($P < 0.05$;
233 Fig 7).

Discussion

This study optimized the key factors for cryopreservation of the Australian flat oyster sperm using the method of liquid nitrogen vapor freezing. The liquid nitrogen vapor method increased both post-thaw motility and PMI by 15% or more compared with a programmable freezing method developed recently [17]. The ability of cryopreserved sperm to fertilize egg is the ultimate criterion for sperm quality assessment but fertilization assessment was not applicable in this species due to lack of a reliable method to obtain high quality eggs. Post-thaw motility and PMI were used as indicators to assess sperm quality in this study because these two variables have strong correlations with fertility in published references [8, 33].

Cryoprotectants might be toxic to sperm with the increase in their concentrations and exposure durations, therefore the selection of a cryoprotectant normally initiates with toxicity evaluation. The suitability of a cryoprotectant is also species specific and a cryoprotectant suitable for one species might be toxic to another in a taxonomic group. Surprisingly, up to 20% of DMSO, EG and PG were non-toxic to the sperm of Australian flat oysters after 90 min equilibration. Although DMSO, EG and PG are suitable for sperm cryopreservation in many marine invertebrates, they are generally toxic at the concentration of 15% or higher and the exposure duration of 30 min or longer [16, 25]. The sperm tolerant limits to these three cryoprotectants are higher in the Australian flat oyster than in other marine invertebrate species reported so far. However, 10% methanol or higher was toxic to this species, which is similar to the toxicity reported in the European flat oyster [39], mangrove oyster [34] and Mediterranean mussel [12].

The permeable cryoprotectants could enter the cell and improve its osmotic balance, thereby reduce the chance of intracellular ice crystal formation, and minimize freezing injury to the cell. A lower concentration and shorter exposure duration of a cryoprotectant might be

insufficient to bring an equilibrium between extracellular and intracellular solutes, but a higher concentration and longer exposure duration might cause a solute effect, i.e., the cell dehydration limit exceeds the solute concentration [31]. Both these consequences are deleterious to sperm survival, therefore delicate adjustment between the osmotic balance and solute effect would provide better protection from freezing injury. In this study, 15% EG, 10% EG and 10% DMSO were suitable cryoprotectant concentrations for the Australian flat oyster sperm. The other cryoprotectant concentrations evaluated in this study were either insufficient to protect sperm or imposed solute and toxic effects. EG was also a suitable cryoprotectant in other marine invertebrates, but the optimum concentration might differ between species, i.e., 15% EG in the European flat oyster [39] and 7% EG in Mediterranean mussel [12]. DMSO is the most widely used cryoprotectant in aquatic species, with the concentration range of 5-20% being found optimal in different aquatic species [30].

Addition of non-permeable cryoprotectants to permeable cryoprotectants could provide better osmotic balance and cell membrane cohesiveness, and minimizes ice crystallization. This strategy has been widely used to improve cryopreserved sperm quality in different animal clades including mammals [1,15], teleosts [10, 35] and invertebrates [2, 26]. In this study, addition of glucose or trehalose to DMSO and EG improved the post-thaw sperm motility, and the combination of 15% EG + 0.2 M trehalose produced the highest sperm motility. In other marine invertebrates, the highest post-thaw motility was achieved with the combination of 8% DMSO + 0.25 M trehalose and 5% DMSO + 0.5 M trehalose in the Chilean oyster [4], 5-12% DSMO + 0.45 M trehalose in the Pacific oyster [5], 5% DMSO + 1 M trehalose in the black-lip pearl oyster [29]. These results suggest that the types and concentrations of permeable and non-permeable cryoprotectants are species specific.

Freezing is the most sensetive step in cryopreservation process. While a faster freezing induces intracelluar ice formation due to insufficient time for excess water to leave

the sperm, a slower freezing causes solute effect due to exposure to the cryoprotectant medium for a longer period. Thereby, a balance is needed for the cell viability. In this study, the height of 8 cm from the liquid nitrogen surface resulted in the highest post-thaw motility and PMI of all the heights evaluated, which differs from the optimal heights reported for other marine invertebrates. For example, the hight of 3 cm was found to be the optimal in the black-lip pearl oyster [22], 5.2 cm in the green-lip and black-lip abalone [27, 28] and 9-12.5 cm in the Japanese pearl oyster species [7, 23]. Athough the optimal height differs among speceis, the results in published literature including this study suggest a narrow range of optimal height among marine invertebrates.

The holding duration on a rack (the period in which samples are placed in liquid nitrogen vapor before being plunged in liquid nitrogen) affect the viability of cryopreserved sperm. The holding duration changes with the endpoint temperature and allows cryoprotectants to equilibrate between inter- and extra-cellular media. The highest post-thaw motility and PMI were achived by holding sperm for 10 min in liquid nitrogen vapor although it was not significantly different from those held for 20 min, suggesting that the holding duration from 10 to 20 min would be suitable for sperm cryopreservation in this species. The period of 10 min holding was also applied for cryopreservation of greeen-lip abalone, black-lip abalone and black-lip pearl oyster [22, 27, 28].

Straw volume is important from both cryobiological and applicational aspects. Regardless of the freezing and thawing procedures, the straw volume determines the actual heat transfer rate. A high-throughput automation for commercial scale application can be achieved with 0.25 ml and 0.5 ml French straws [40, 41] but a larger volume (e.g., 4.5 ml cryovials) has greater application in small and medium scale hatchery operations where such automation is unavailable [3]. Interestingly, the effect of straw size in cryopreservation differs among studies, probably due to the interaction of other factors such as freezing and

thawing rates. In this study, there was no significant difference in post-thaw sperm motility and PMI between sperm samples cryopreserved in 0.25 ml and 0.5 ml straws. However, significantly higher post-thaw motility and PMI were achieved in 0.5 ml straws compared with those in 0.25 ml straws in this species using a programmable freezing method [17]. Straw volume has no significant effect on post-thaw sperm motility in the Pacific oyster as well [13]. In contrast, significant differences in post-thaw sperm motility were found between straw volumes in rainbow trout and Japanese pearl oyster [6, 9].

Although freezing methods could affect sperm viability [38], this aspect has rarely been investigated in marine invertebrates. Sperm cryopreservation protocols have been developed from independent studies using a range of freezing methods including laboratory-scale programmable freezing [12], commercial-scale programmable freezing [14, 41], liquid nitrogen vapor freezing [28], and methanol - dry ice [5]. In comparions between freezing methods, liquid nitrogen vapor produced significantly higher post-thaw motility and PMI than those cryopreserved with programmable freezing in this study. Although both programmable freezing and liquid nitorgen vapour freezing have produced high post-thaw motility and fertility in marine molluscs [25], the results of this study indicate a higher sensitivity of flat oyster sperm to programmable freezing.

In conclusion, 62.2% post-thaw motility and 67.3% PMI were achieved in this study using the liquid nitrogen vapor method. The protocol requires that sperm are equilibrated in 15% EG + 0.2 M trehalose for 20 min, packaged in 0.25 ml straws, placed on a foam rack of 8 cm above the liquid nitrogen surface for 10 min, and then immediately plunged into liquid nitrogen. By comparing the post-thaw motility and PMI achieved in programmable freezing technique, the study concludes that the liquid nitrogen vapor freezing technique is an effective method for the cryopreservation of Australian flat oyster sperm.

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Figure legends

Fig 1: Motility (%) of sperm after exposure to different concentrations of dimethyl sulfoxide (DMSO), methanol (met), ethylene glycol (EG) and propylene glycol (PG) for 30, 60 or 90 min equilibration. The differences in sperm motility exposed to DMSO, EG and PG were not significant ($P > 0.05$). Different letters in methanol represent significant effect of cryoprotectant concentrations and exposure durations on sperm motility ($P < 0.05$). Each bar represents mean ± SE of three replicates.

Fig 2: Post-thaw motility (%) of sperm cryopreserved with different cryoprotectant concentrations (A) permeable cryoprotectants and (B) combinations of permeable and non-permeable cryoprotectants. Each bar represents mean ± SE of four replicates. Different letters represent significant effect of cryoprotectants on sperm motility. DMSO, dimethyl sulfoxide; EG, ethylene glycol.

Fig. 3: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm cryopreserved at different equilibration durations. Each bar represents mean ± SE of four replicates. Different letters represent significant effect of equilibrium durations on sperm motility or PMI.

Fig 4: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm cryopreserved at different distances from liquid nitrogen. Each bar represents mean ± SE of five replicates. Different letters represent significant distance effect on sperm motility or PMI.

Fig 5: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm frozen at different durations in liquid nitrogen vapour. Each bar represents mean ± SE of four

replicates. Different letters indicate significant effect of holding durations on sperm motility or PMI.

Fig 6: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm cryopreserved in 0.25 ml and 0.5 ml French straws. Each bar represents mean ± SE of four replicates.

Fig 7: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm cryopreserved with programmable freezing and liquid nitrogen vapor methods. Each bar represents mean ± SE of three replicates. Different letters indicate significant effect of freezing methods on sperm motility or PMI.

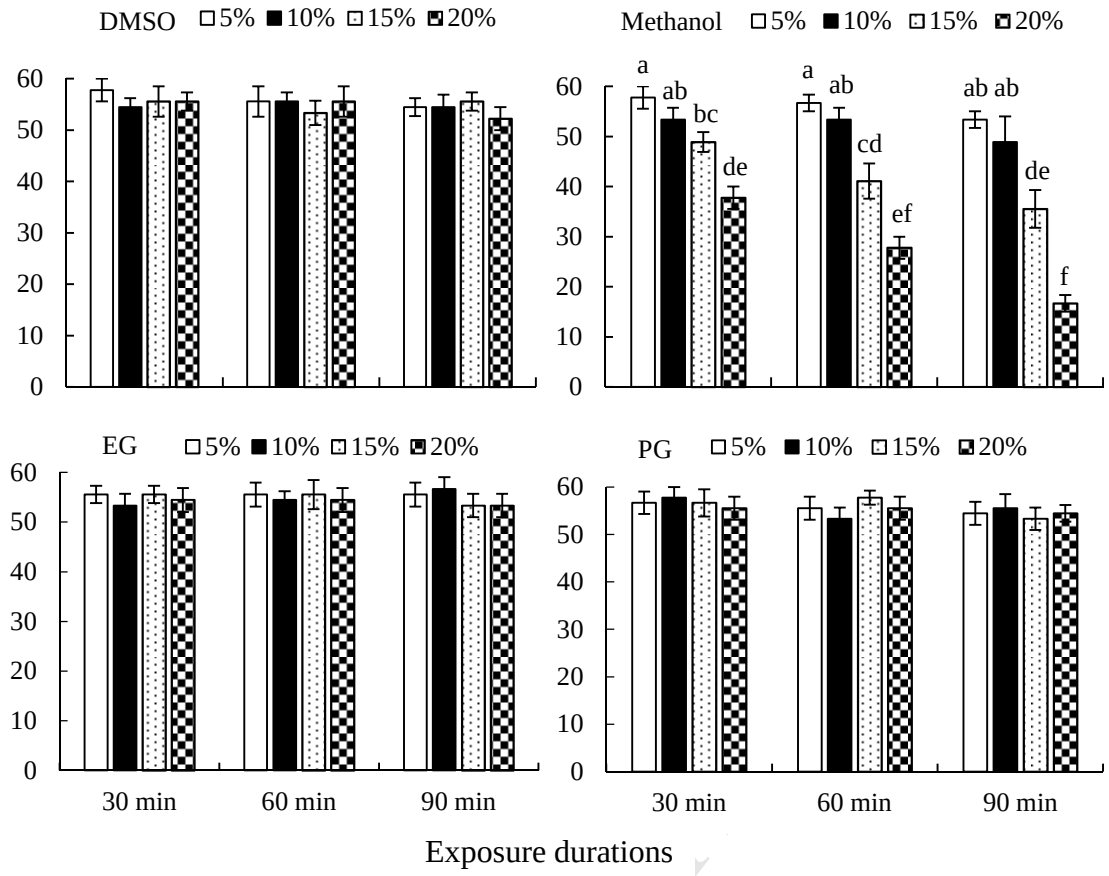

Figure 1

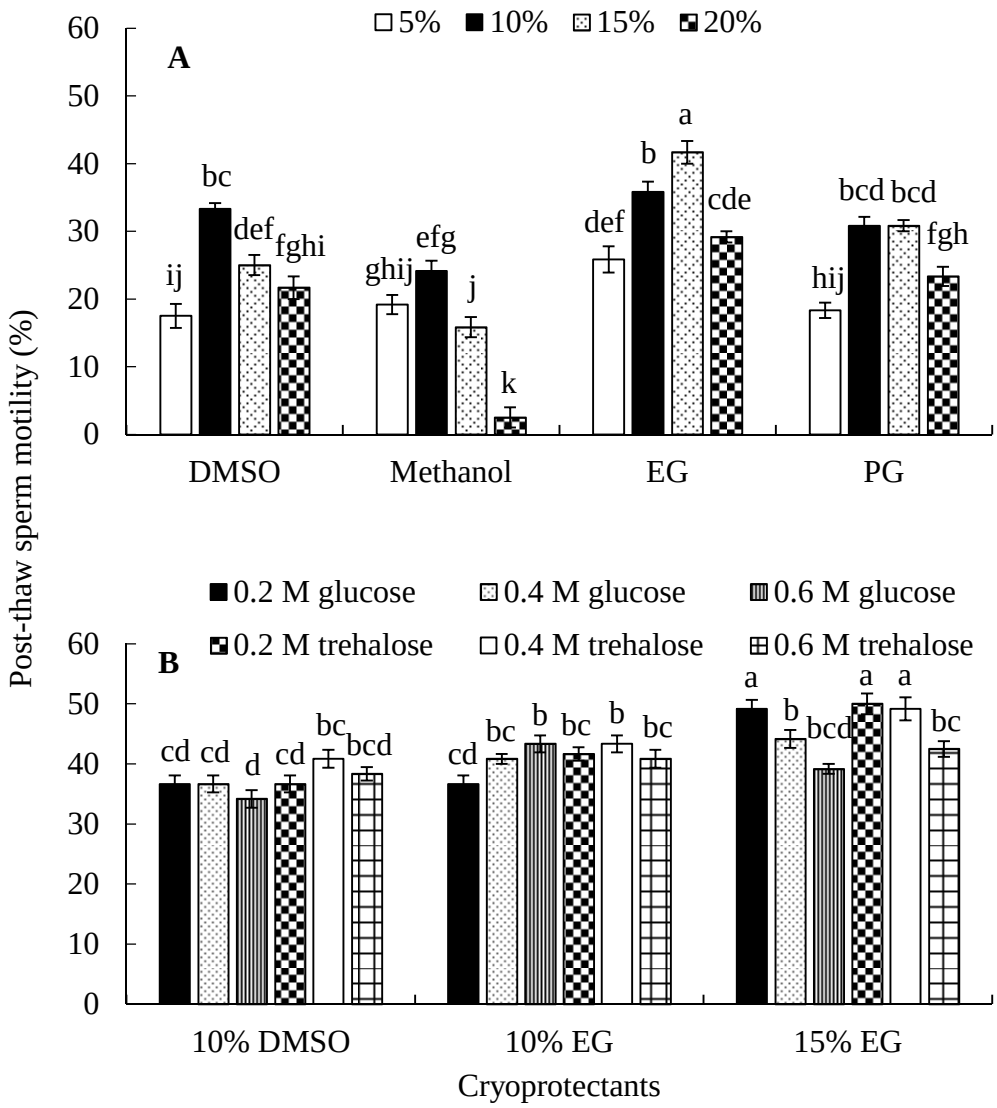

Figure 2

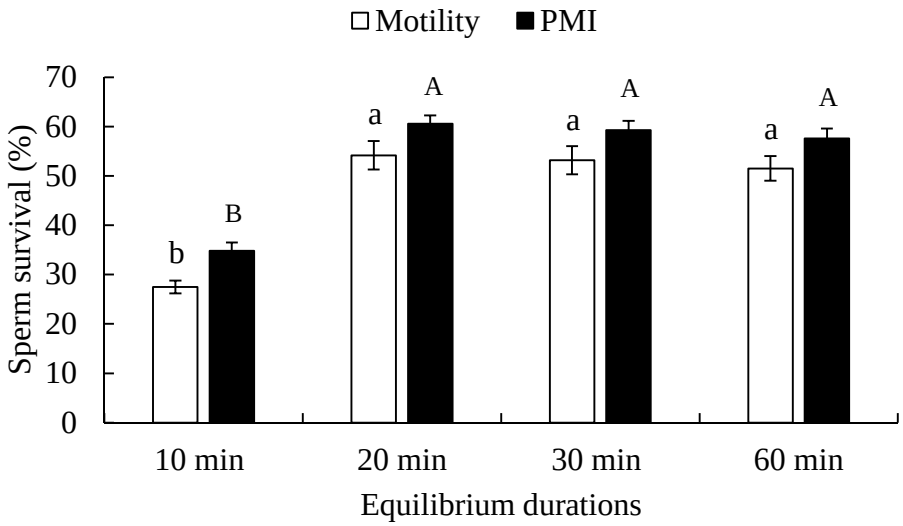

Figure 3

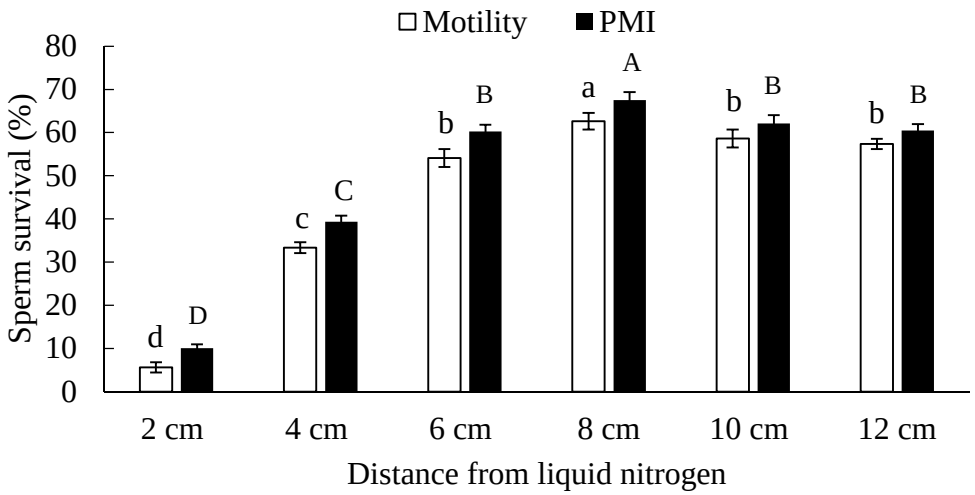

Figure 4

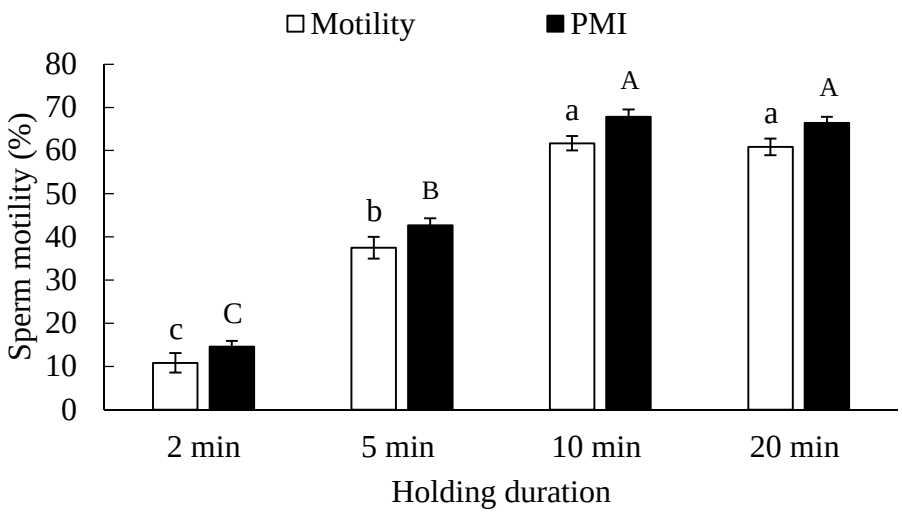

Figure 5

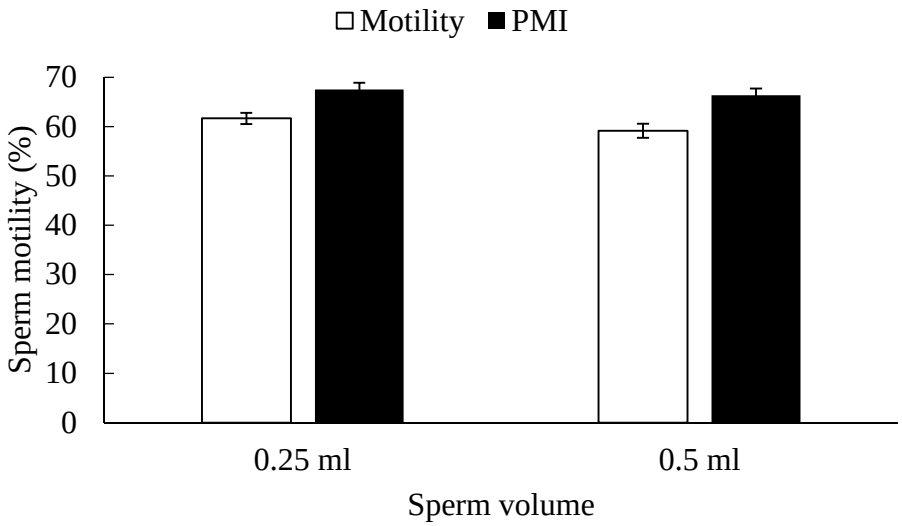

Figure 6

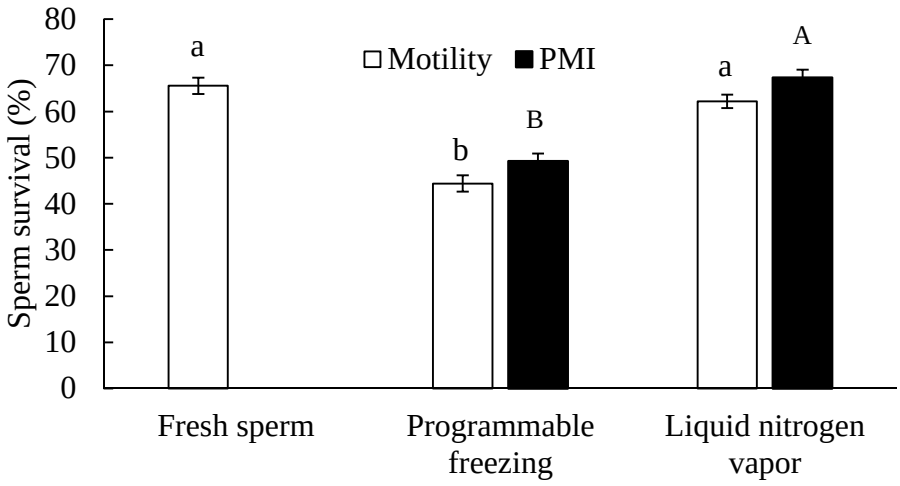

Figure 7

Highlights

A simple method was developed for cryopreservation of *Ostrea angasi* sperm using liquid nitrogen vapour

A combination of 15% ethylene glycol + 0.2 M trehalose produced higher sperm survival

Holding sperm 8 cm above liquid nitrogen surface for 10 min produced higher sperm survival

The post-thaw sperm survival was improved by liquid nitrogen vapour freezing compared with programmable freezing